Inhibition of Human Skin Phospholipase A_2 by "Lipocortins" Is an Indirect Effect of Substrate/Lipocortin Interaction

Boris Christoph Bastian,* Corinna Sellert,* Axel Seekamp,* Jürgen Römisch,† Eric-Paul Pâques,† and Eva-Bettina Bröcker*

*Department of Dermatology, University of Würzburg Medical School, Würzburg; and †Behringwerke AG Research Laboratories, Marburg, Germany

Proteins of the annexin/lipocortin family have been claimed to mediate the anti-inflammatory action of glucocorticosteroids by the inhibition of phospholipases A_2. This hypothesis has been challenged by the finding that annexins do not directly interact with the enzyme in a classical enzyme/inhibitor behavior, but more likely block the access of the phospholipase A_2 to its substrate by binding to phospholipids. Because former studies with skin phospholipase A_2 suggested a specific regulation by annexin-1, we investigated the substrate dependence of this effect. For this purpose phospholipase A_2 activities in human epidermis and dermis homogenates were measured in the presence of various amounts of annexins-1, -2, or -5. The respective annexin was preincubated in separate series either with the substrate or with the enzyme. We found a partial inhibition of both epidermal and dermal phospholipase A_2 activities with all annexins tested (annexin-5 $\gg$ annexin-2 > annexin-1). The inhibitory effect was absolutely dependent on the annexin/phospholipid ratio and occurred only at very high annexin concentrations relative to the amount of substrate.

Our data demonstrate that the inhibition of human skin phospholipase A_2 by annexins depends on the substrate concentrations, as has been shown for phospholipases A_2 of other origins as well. All observations can be explained by the current "substrate depletion model" characterizing the indirect effects of annexins on phospholipase A_2 activities. It is therefore rather unlikely that annexins are directly involved in the regulation of phospholipase A_2 activity of human skin under physiologic conditions. Key words: Phospholipase A_2/annexins/corticosteroids. *J Invest Dermatol 101:359–363, 1993*

Phospholipases A_2 (EC 3.1.1.4) are a group of enzymes that hydrolyze the ester bond of fatty acids from the *sn*-2-position of phospholipids. The release of arachidonic acid from membranes by phospholipases A_2 and its subsequent conversion into leukotrienes, prostaglandins, and other eicosanoids is an important catalytic event in the process leading to inflammation. Additionally, phospholipases A_2 are involved in the biosynthesis of platelet-activating factor (PAF). Based on amino acid sequence data, phospholipases A_2 have been divided into two groups [1]. Group I encompasses pancreatic, Elapidae (cobra), and Hydrophidae (sea snake) venom enzymes. Group II enzymes are found in Crotalidae and Viperidae and contain at the carboxy terminal of the molecule an extension of six or seven residues that are absent in group I phospholipases A_2. Extracellular phospholipases A_2, which are regarded to be involved in inflammation, have been found to belong to group II. Recently, a third type of phospholipase A_2 with a molecular weight of 85 kD has been characterized [2,3]. This enzyme is found in the cytosol and is active at the low calcium concentrations present in the cytosol. It has been claimed to hydrolyze specifically phospholipids that have arachidonic acid esterified at their *sn*-2 position and it is possibly involved in the release of eicosanoids and intracellular signaling.

Although a variety of agents block either prostaglandin or leukotriene production, compounds that inhibit both pathways, such as the glucocorticoids, have proved to be the most potent anti-inflammatory agents. During the last 10 years a group of structurally related, calcium-dependent phospholipid-binding proteins has been discovered (see [4] for review). These proteins had been shown by numerous investigators to be inducible by glucocorticoids and to inhibit phospholipase A_2 activity *in vitro* (see [5] for review). These independent observations led to the hypothesis that the inhibition of phospholipase A_2 by this group of proteins is the mechanism of the anti-inflammatory action or glucocorticoids. Based on this suggested mode of action they were initially termed lipocortins. Later, they were renamed using the more general term annexin [6].

In the last few years, the idea that the proteins of the annexin/lipocortin family are physiologic regulators of phospholipases A_2 has been challenged by several observations. First it became obvious that not only phospholipase A_2 but also phospholipase C and phospholipase D are inhibited by "lipocortin" under similar assay conditions [7]. Later, this inhibition was shown to be dependent on the assay conditions, i.e., the concentration of the substrate [8,9]. Finally, the hypothesized regulatory link between the synthesis of annexin-1 and glycocorticoids on a pre-transcriptional level could not be demonstrated by all investigators [10,11]. In short, these findings placed the "lipocortins" beyond the scope of their original definition as being i) glycoproteins whose ii) synthesis or secretion is

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Reprint requests to: Dr. Boris Bastian, Hautklinik der Universtät Würzburg, Josef-Schneider-Str. 2, D-7080 Würzburg, Germany.

Abbreviations: BSA, bovine serum albumin; DMSO, dimethylsulfoxide; PAF, platelet-activating factor.

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stimulated by glucocorticoids and that iii) specifically inhibit phospholipase A_2. Therefore, these related proteins are now referred to as annexins [6].

Phospholipase A_2 activity has been found in human skin, and raised levels have been described in lesional and non-lesional psoriatic skin. Thus, the release of arachidonic acid by epidermal phospholipase A_2 and its subsequent conversion to leukotriene B_4 and other lipid mediators could represent a pivotal mechanism in the mediator cascade leading to inflammation and immigration of granulocytes [12]. Phospholipase A_2 activity in human epidermis has been claimed to be specifically inhibited by lipocortin I/annexin-1 even at high substrate concentrations [13], suggesting a distinct behavior of human skin phospholipase A_2 in comparison to other phospholipases A_2 [8,9]. The hypothetic regulation by annexins to characterize human skin phospholipase A_2 or to monitor topical steroid action by the measurement of phospholipase A_2 has also been used [14,15]. In the following study, we wanted to investigate the influence of the substrate concentration on the inhibition of human skin phospholipase A_2 by annexins. Furthermore, we asked whether the inhibition of skin phospholipase A_2 is specific to annexin-1, or whether other annexins inhibit human skin phospholipase A_2 as well.

MATERIALS AND METHODS

Tissue Preparation Full-thickness skin was obtained from human cadavers during the first 24 h post-mortem and carefully freed from adjacent fat lobules. After incubation in 2 M potassium bromide for 30 min at 37°C, specimens were rinsed with an ice-cold 0.9% (w/v) NaCl solution for 10 min. The epidermis was separated from the dermis with fine forceps, and the dermal and epidermal fractions were collected on ice. After the tissue had been dried with paper towels, the total weight was determined, and the specimens were snap frozen with liquid nitrogen and stored at −80°C in plastic tubes. Tissue homogenization was carried out in a 0.9% (w/v) NaCl solution on ice in the presence of 1 mM phenylmethylsulphonyl fluoride with a Turrax homogenizer, and subsequently with an all-glass Potter grinder. After centrifugation of the homogenates at 10,000 × *g* for 15 min, the supernatants were stored at −80°C for further processing. Determination of protein was done according to Bradford [16].

Isolation of the Annexins Annexins were purified as described previously [17]. Briefly, freeze-dried human placenta was extracted with buffer containing citrate. After removal of the cell debris the annexins were purified by chromatography on phenyl, (calcium) heparin, and Q-Sepharose, yielding a >95% pure preparation.

Phospholipase A_2 Assay 1-palmitoyl-2-[1-^{14}C]-arachidonyl-*sn*-glycero-3-phosphoethanolamine (New England Nuclear, Boston, MA) was diluted with 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (Sigma, St. Louis, MO) to the desired substrate concentrations and specific radioactivities. The lipid mixture was dried under nitrogen and dissolved in water. This emulsion was sonified two times for 1 min with 60 W under a constant flow of nitrogen. Then the buffer containing 100 mM Tris-HCl, pH 7.0, and 10 mM Ca^{++} was added. Three different substrate emulsions with respective phospholipid concentrations of 2 μM, 10 μM, and 200 μM were prepared. In these emulsions the unlabeled dipalmitoyl-phosphatidylethanolamine was the predominant phospholipid (0.27%, 2.7%, and 13.6%, respectively). Due to the high melting point of the dipalmitoyl-phosphatidylethanolamine, the physical state of the phospholipids is a bilayer. All experiments at a substrate concentration of 2 nM were also performed with 1-palmitoyl-2-[1-^{14}C]-arachidonyl-*sn*-glycero-3-phosphoethanolamine alone to exclude an interference of the relatively higher amount of this phospholipid in this preparation. No significant difference in the inhibition patterns was found. For each substrate the linearity of hydrolysis was determined and experiments were carried out within this range.

Annexins-1, -2, and -5 were diluted with 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, BSA 0.1% (w/v) to varying concentrations, as specified in the legends of the figures. The annexins were preincubated either with the tissue homogenates (assay A) or with the substrate emulsion (assay B) for 15 min at 37°C. The assay was started by the addition of the substrate (assay A) or the homogenates of epidermis or dermis, respectively (assay B). After a maximal incubation time of 15 min at 37°C, the reaction was stopped, and released [^{14}C]-arachidonate was determined by a modified Dole extraction procedure as described [18]. The activity was expressed in mU/ml, reflecting the amount of arachidonic acid in μmol released by 1 ml homogenate per minute. Inhibition was expressed as the percentage of phospholipase A_2 activity compared to a control containing no annexin. All experiments, including blanks containing neither enzyme nor annexins, were carried out in duplicate.

For the statistical analysis the test for the equality of slopes according to Draper and Smith was applied. A $p < 0.05$ was regarded as statistically significant.

RESULTS

The tissue homogenates used had a specific phospholipase A_2 activity of 24 mU and 13 mU per mg of protein for the epidermal and dermal homogenates, respectively (means of four different experiments). Initial inhibition studies at a substrate concentration of 200 μM and an annexin concentration of 27 nM showed no clear effect of the three annexins added, independent of the preincubation procedure of the annexins. Only annexin-5 decreased the enzyme activity by 10% in the dermal fraction and 30% in the epidermal fraction, when it was preincubated with the substrate (data not shown). In subsequent experiments we continuously decreased the assay concentration of the substrate to study the effect of lower substrate/annexin ratios. The lowest substrate concentration tested was 2 μM, and here an inhibition of 95% and 75% could be demonstrated with 270 nM annexin-5 and epidermis and dermis, respectively (Fig 1*B*). Annexin-1 and annexin-2 displayed maximal inhibitions of 45% and 65% with dermis and 57% and 72% with epidermis under these conditions. These effects gradually decreased with rising substrate concentrations for all annexins (Fig 1*A,B*).

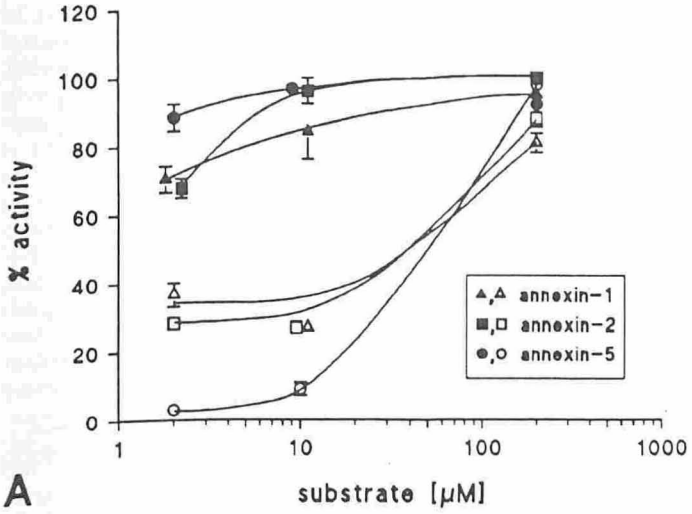
To investigate the influence of the incubation procedure we ran separate series, preincubating the annexin either with the substrate or with the tissue homogenate. When the annexins were preincubated with the substrate (Fig 1*B*) the inhibition was stronger than the inhibition found after preincubation with the enzyme (Fig 1*A*). In the dermis this effect reached a significant level only for annexin-2 ($p = 0.001$) and annexin-5 ($p = 0.012$). For the epidermal fraction this dependency on the incubation procedure only became apparent with annexin-5 ($p = 0.004$).

Under constant substrate concentrations the inhibitory effect was found to be dependent on the concentration of the respective annexin. Figure 2 shows that annexin-5 is about 100 times more effective than annexin-1 and annexin-2 in inhibiting both tissue homogenates. Fifty percent of the activity of the epidermal phospholipase A_2 was inhibited in the presence of 3.5 pmol/ml of annexin-5, whereas 200 pmol/ml of annexin-1 or annexin-2 were needed to obtain the same effect. These inhibition profiles of the three annexins were comparable to those obtained with dermal phospholipase A_2 where, due to the overall weaker inhibition of this fraction, 50% inhibition values for annexin-1 and annexin-2 were not determined.

As can be seen in Figs 1 and 2, the inhibition profiles of the epidermal and dermal phospholipase A_2 differed. Under the same assay conditions epidermal phospholipase A_2 was inhibited more strongly than dermal phospholipase A_2 at low substrate concentrations (Fig 1). But these difference were only significant when the annexins were preincubated with the enzyme (Fig 1*A*) with $p = 0.012$ for annexin-1, $p = 0.025$ for annexin-2, and $p = 0.0005$ for annexin-5.

The correlation of the enzyme activity with the molar ratio of substrate and annexin showed the phospholipase A_2 inhibition of all annexins to be a function of the quantitative relation of substrate and annexin molecules (Fig 3). Here again, the higher inhibitory potential of annexin-5 is obvious, whereas annexin-1 and annexin-2 were similar in their inhibition profiles.

To investigate whether the inhibitory effect also depends on the amount of enzyme, we incubated annexin-5 at a concentration of 27 nM with increasing amounts of the homogenates of epidermis and dermis, as depicted in Fig 4. In this experiment the amount of inhibition decreased with rising concentration of the enzyme added, suggesting a competition of phospholipase A_2 and annexin for the binding to the substrate (Fig 4).



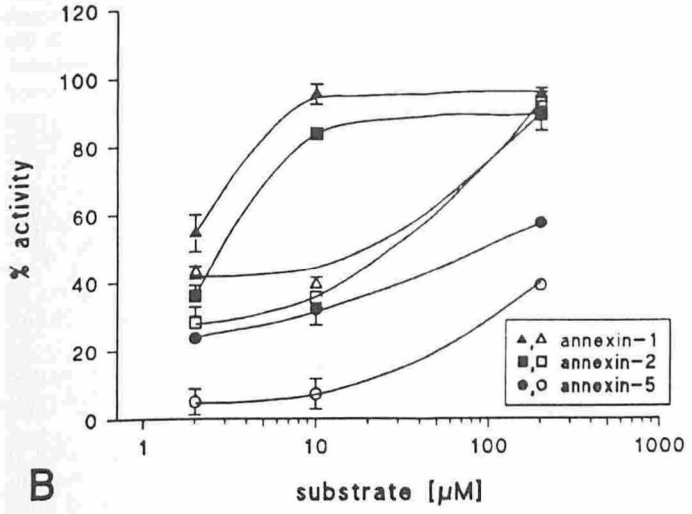
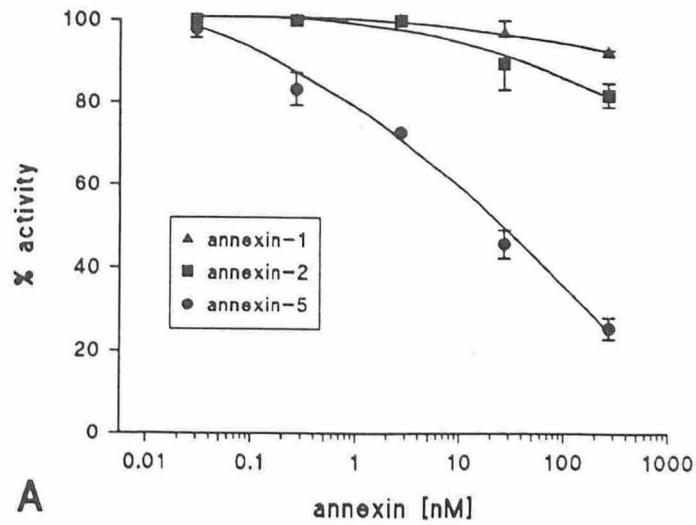


Figure 1. Phospholipase A_2 activity in homogenates of dermis *(closed symbols)* and epidermis *(open symbols)* in the presence of annexin-1, annexin-2, or annexin-5 at different substrate concentrations. Values represent means of three independent experiments performed in duplicate. *Error bars,* SEM; data points lacking error bars have a SEM below 2%. To avoid any overlaps, in some cases only one-half of the error bar is displayed. Annexins were pre-incubated at a concentration of 270 nM with the enzyme *(A)* or with the substrate *(B)*.



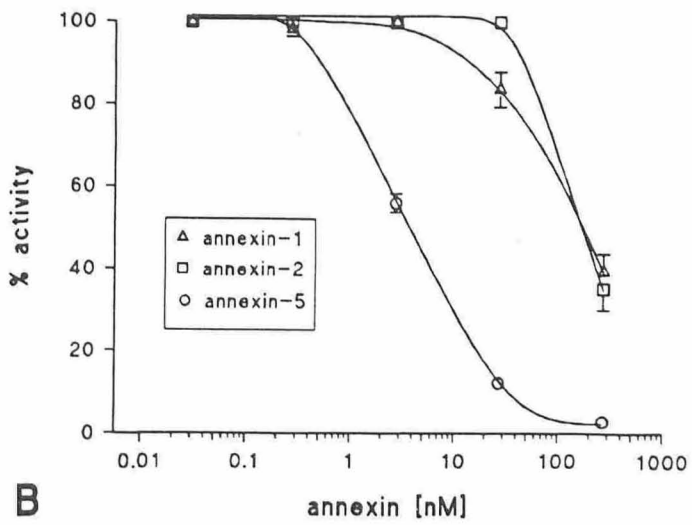


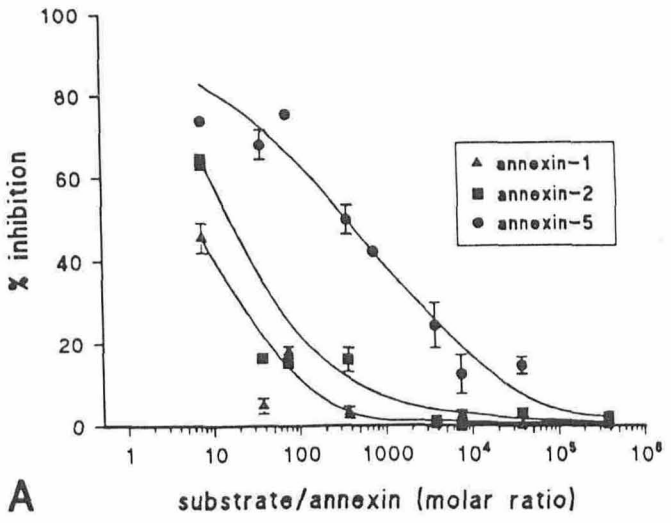
Figure 2. Phospholipase A_2 activity in homogenates of dermis *(A)* and epidermis *(B)* in the presence of rising concentrations of annexins-1, -2, or -5. Annexins were pre-incubated with the substrate at a concentration of phosphatidylethanolamine of 10 μM. Values represent means of two independent experiments performed in duplicate. *Error bars,* SEM; data points lacking error bars have a SEM below 2%. To avoid any overlaps, in some cases only one-half of the error bar is displayed.

DISCUSSION

In this study we demonstrate that an inhibitory effect of annexins on phospholipase A_2 of human skin occurs only at high annexin/phospholipid ratios. Hence, phospholipase A_2 activity in human skin homogenates shares this property with other phospholipases A_2 like rat liver mitochondrial phospholipase A_2 [8] and porcine pancreatic phospholipase A_2 [9].

From the observed influence of the substrate concentration and the finding that the annexin does not bind to the enzyme, but much more tightly to the substrate [19], the so-called 'substrate depletion model' of the annexin action had been derived [9]. According to this model, the annexin binds to the phospholipid substrate, forming a substrate-inhibitor complex. This complex is especially tight for the interaction with anionic substrates and depends on the presence of calcium ions. By this substrate-inhibitor interaction, the access of phospholipase A_2 to its substrate is impaired, and a competition of phospholipase A_2 and annexin for the substrate results. This is in accordance with the difference of the inhibition patterns in dependence on the incubation mode in our study (Fig 1). Only if the substrate concentration is sufficiently low relative to the amount of annexin does an inhibitory effect become apparent. Considering the fact that only a restricted fraction of the phospholipids of the micelles can be hydrolyzed by phospholipases, the effective ratio of annexins to substrate might even be lower than depicted in Fig 3. Under physiologic conditions such ratios are highly unlikely to occur. *In vivo* substrate is not a limiting factor, and very high concentrations of annexins would be necessary to give any biologic effect.

A potentiation of the *in vitro* effect of annexins can occur in the



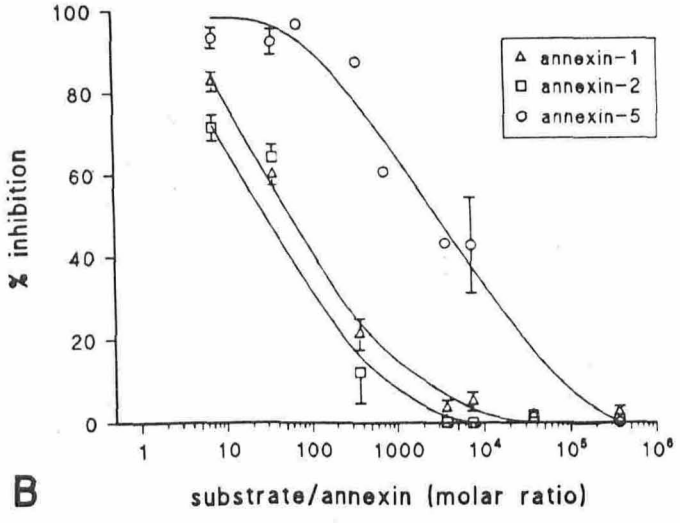


Figure 3. Inhibition of phospholipase A_2 activity in homogenates of dermis *(A)* and epidermis *(B)* as a function of the molar ratio of substrate and annexin-1, -2, and -5. Annexins were pre-incubated with the substrate. *X-axis,* molar ratio of substrate to annexin. The absolute ratios of substrate to annexin for the individual data points are: x = 7.4 (2 μM/270 nM); x = 37 (10 μM/270 nM); x = 74 (2 μM/27 nM); x = 370 (10 μM/27 nM); x = 740 (200 μM/270 nM); x = 3700 (10 μM/2.7 nM); x = 7400 (200 μM/27 nM); x = $3.7 \cdot 10^4$ (10 μM/0.27 nM); x = $3.7 \cdot 10^5$ (10 μM/0.027 nM).

presence of deoxycholate in the assay mixture. Deoxycholate is necessary when phosphatidylcholine (PC) is used as substrate in phospholipase A_2 assays and can form complexes with the calcium in the assay mixture. In the presence of annexins, which will also need calcium ions to bind to the phospholipids, the calcium concentration can become critically low. This depletion of calcium can decrease phospholipase A_2 activity, which is absolutely calcium dependent. Therefore, deoxycholate/PC mixtures as phospholipase A_2 assay systems are regarded as unsuited to study the effects of annexins [9,20]. Other investigators omitted additional calcium from the assay mixture, so that a competition between annexins and phospholipase A_2 for calcium in the assay could have occurred.

Our results are contradictory to the finding of Cartwright *et al* [13]. We could not find any significant inhibition of epidermal phospholipase A_2 by annexin-1 above a ratio of substrate/annexin of

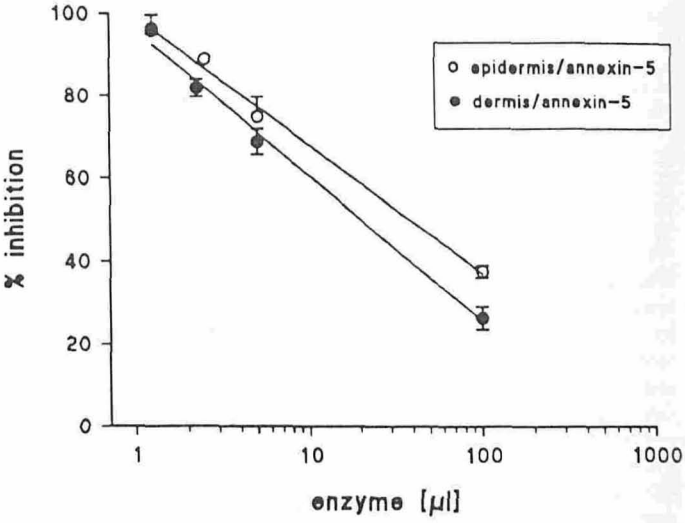


Figure 4. Inhibition of phospholipase A_2 activity in dermis and epidermis homogenates in the presence of 27 nM annexin-5 in dependence of the amount of homogenate added. The annexin was pre-incubated with the substrate at a concentration of phosphatidylethanolamine of 50 μM. Values represent means of two independent experiments performed in duplicate. *Error bars,* SEM; data points lacking error bars have a SEM below 2%. To avoid any overlaps, in some cases only one-half of the error bar is displayed.

50. This difference might be due to the above-mentioned problematic nature of the deoxycholate/PC mixture used in their study. The 50% inhibitory concentration of annexin-1 in our study is in accordance with the findings of authors who investigated phospholipase A_2 from other sources‡ [8,21]. Interestingly, annexin-5 displayed a 100 times higher inhibitory potential than annexin-1. This is in accordance with the very potent anti-coagulatory effects of annexin-5 *in vitro.* Because annexin-5 has a strong binding affinity to phospholipid membranes, comparable to that of the factors Va and Xa to platelet membranes [22], it is able to compete for activation surfaces. Whether this anticoagulant mechanism is of physiologic relevance is, however, questionable [23].

Since the investigation of Ziboh *et al* [24,25], human skin is known to contain considerable phospholipase A_2 activity. Later on, raised levels of phospholipase A_2 were found in association with inflammatory skin disorders, i.e., psoriasis vulgaris [26]. Up to now, no attempt has been made to characterize the phospholipase A_2 activity found in human epidermis with regard to the possible groups of phospholipases A_2 stated above. Studies on other inflammatory disorders like rheumatoid arthritis made it likely that at least the majority of the phospholipase A_2 activity found in human skin can be assigned to a group II phospholipase A_2 [27]. This assumption is substantiated by the finding that antibodies against pancreatic phospholipase A_2 do not crossreact with phospholipase A_2 from human skin, and that human epidermal phospholipase A_2 differs from human pancreatic phospholipase A_2 in solubility and pH optimum.§¶ By analyzing the inhibition patterns of annexin-1 with skin phospholipase A_2, it has been concluded that homogenates of human epidermis may contain different phospholipases A_2. Bergers *et al* described a phospholipase A_2 activity in the upper epidermis that could not be modulated by pretreatment with corticosteroids or by the addition of alkaline phosphatase, indirectly implicating a lack

‡ Davidson FF, Lister MD, Glaser KB, Dennis EA: Analysis of the inhibition of phospholipase A_2 by lipocortins and calpactins (abstr). *J Cell Biol* 107:425, 1989.

§ Bastian BC, Seekamp AW, Burg G: Measurement of human epidermal and dermal phospholipase A_2 using anionic substrates (abstr). *J Invest Dermatol* 96:627,1991.

¶ Seekamp AW, Franz S, Burg G, Bastian BC: Partial characterization and immunohistochemical localisation of phospholipase A_2 of human skin (abstr). *J Invest Dermatol* 96:1010, 1991.

of regulation by annexin-1 [14]. In this study an artificial assay system with 30% dimethylsulfoxide was used. In the presence of 30% dimethylsulfoxide, phospholipids do not form bilayers but give a solution of monomers. According to the substrate depletion model described above, annexins would probably not be able to bind to phospholipids in monomeric solutions and thereby become unable to inhibit phospholipase A_2 activity. Moreover, no controls for a positive modulation by alkaline phosphatase under these assay conditions were used. Therefore, it remains unclear whether the lack of regulation by annexins was a specific attribute of the phospholipase A_2 studied or due to the assay conditions used. In short, the growing body of evidence that the characteristic of being inhibited by annexins is not a specific criterion for phospholipases A_2 makes this approach unsuited for the classification of phospholipases A_2. Evidence for the existence of different phospholipase A_2 in human epidermis has also been derived from the observation that the inhibition of human epidermal phospholipase A_2 by annexins is not complete. From this finding, Cartwright *et al* postulated the presence of an annexin-sensitive and a non-sensitive form of phospholipase A_2 in human epidermis [13]. But experiments with pure phospholipase A_2 of different sources also showed a maximal inhibition of 70% [8]. Disregarding the problems of the phospholipase A_2 assay used in this study, as stated above, this incomplete inhibition alone does not prove the existence of a second phospholipase A_2. Thus, evidence for the presence of several biochemically different A_2 phospholipases in human epidermis is still lacking.

In our study, the inhibition profiles of the dermal and epidermal homogenates showed significant differences. The epidermal fraction was inhibited at lower annexin concentrations than the dermal fraction. Whether this is due to a true difference in the phospholipases of both fractions or to additional factors present in the homogenates remains to be established in further experiments with purified enzymes.

Annexins-1 and -2 are abundantly present in human epidermis and dermis [28], and an increased expression has been found in well-differentiated squamous cell carcinomas and in benign hyperproliferative skin disorders.** Their definite function still remains unclear. Analysis of the tertiary structure of annexin-5 revealed a symmetric molecule with ion-channel characteristics. The amphipathic annexins can probably partially penetrate the membrane and channel ions in a still unknown way [29,30]. Annexins may thus in some way play a role in inflammation, growth regulation, or oncogenesis, but it appears unlikely that they are directly involved in the regulation of phospholipase A_2.

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